Cytomorphological and molecular diversity in backcross-derived inbred lines of sunflower (*Helianthus annuus* L.)

M. Sujatha, A.J. Prabakaran, Sangam L. Dwivedi, and S. Chandra

Abstract: A set of 250 distinct, stable, and uniform backcross-derived inbred lines were developed in sunflower through 5 interspecific cross combinations involving 4 wild diploid annual species (*Helianthus argophyllus*, *H. petiolaris*, *H. annuus*, and *H. debilis*). The presence of the wild-species genome in these inbred lines was confirmed through higher chromosome associations (tri- and quadrivalents) at diakinesis. Maximum structural rearrangements of chromosomes were observed in lines derived from *H. petiolaris*. Forty morphologically diverse inbred lines along with 2 controls were subjected to measurements of phenotypic and genetic distance using 118 simple sequence repeat (SSR) markers of known map location. A total of 204 alleles were identified and the number of alleles per locus varied between 2 and 5. There were 46 unique alleles and the number of unique alleles was highest in the lines derived from the cross involving *H. petiolaris.* The polymorphism information content (PIC) values ranged from 0.05 to 0.575. The pair-wise comparison values based on genetic dissimilarity estimates computed using molecular marker data varied between 0.143 and 0.486 among the 42 lines. The results indicate that the sunflower gene pool could benefit from introgression of novel alleles from the latent genetic diversity present in the wild species and particularly through exploitation of the diploid annual *H. petiolaris.*

Key words: chromosome associations, diakinesis, diploid, *Helianthus* spp., genetic distance, interspecific hybridization, introgression, prebreeding, simple sequence repeats.

Résumé : Un jeu de 250 lignées fixées dérivées de retrocroisements, toutes différentes, stables et uniformes, ont été produites chez le tournesol par le biais de cinq croisements interspécifiques impliquant quatre espèces annuelles diploïdes sauvages (*H. argophyllus*, *H. petiolaris*, *H. annuus* et *H. debilis*). La présence d'ADN des espèces sauvages au sein de ces lignées fixées a été confirmée par l'observation d'associations chromosomiques, tels des tri- et quadrivalents, à la diacinèse. Les réarrangements structuraux les plus importants ont été observés chez les lignées dérivées de croisements impliquant le *H. petiolaris*. Sur la base de la diversité morphologique, 40 lignées fixées différentes et deux témoins ont fait l'objet d'analyses phénotypiques et de distance génétique, cette dernière à l'aide de 118 marqueurs SSR de position connue. Au total, 204 allèles ont été observés et le nombre d'allèles par locus variait entre 2 et 5. Il y avait 46 allèles uniques et le plus grand nombre d'allèles uniques a été observé chez les lignées dérivées du croisement avec le *H. petiolaris*. L'indice de polymorphisme (PIC) variait entre 0,05 et 0,575. Les valeurs obtenues lors de la comparaison des paires de lignées sur la base des estimés de dissimilarité génétique, obtenus grâce aux marqueurs, variaient entre 0,143 et 0,486 parmi les 42 lignées. Ces résultats indiquent que le pool génique du tournesol pourrait bénéficier de l'introgression d'allèles nouveaux tirés de la diversité génétique latente présente au sein des espèces sauvages, particulièrement via l'exploitation de l'espèce diploïde annuelle *H. petiolaris*.

Mots-clés : associations chromosomiques, diacinèse, espèces diploïdes d'*Helianthus*, distance génétique, hybridation interspécifique, introgression, amélioration primaire, microsatellites.

[Traduit par la Rédaction]

Introduction

Worldwide, sunflower (*Helianthus annuus* L.) is fourth in importance as an oilseed after soybean, groundnut, rapeseed, and mustard and is grown on 23.4 million ha with a total annual production of 31.1 million tons and an average yield of 1.32 t ha^{-1} (FAO 2005). It is native to North America and has spread across the globe owing to its wide adaptability and short growing season. The commercial sunflower has a narrow genetic base and as a consequence only a few introductions constitute the base material for the development of new cultivars or hybrids. For example, only 10 to 12 introductions were used in India to develop new cultivars or hybrids (Sujatha et al. 2006).

Interspecific hybridization has played a pivotal role in the genetic improvement of sunflower (Korell et al. 1996). In-

Received 7 July 2007. Accepted 23 January 2008. Published on the NRC Research Press Web site at genome.nrc.ca on 13 March 2008.

Corresponding Editor: P. Gustafson.

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 doi:10.1139/G08-008

terest in interspecific hybridization in sunflower is mainly for transfer of characters such as disease and insect resistance, salt tolerance, drought tolerance, fatty acid composition, protein quality, cytoplasmic male sterility, and fertility restoration. Use of related wild sunflower germplasm has allowed breeders to obtain resistance to numerous diseases, develop several cytoplasmic sources, and increase seed and oil yields (Korell et al. 1996). Forty-nine wild *Helianthus* species are available, which fall into 2 growth habit groups (annual and perennial) and 3 ploidy groups (diploid, tetraploid, and hexaploid). Among these, the diploid annuals constitute the primary gene pool and are mostly cross-compatible with cultivated sunflower.

The need to broaden the genetic base of cultivar germplasm, break the yield stagnation, develop material for diverse situations, and introgress specific characters from wild *Helianthus* species raised the interest in a pre-breeding programme in India. The backcross-derived inbred lines exhibited wide variability in terms of several distinct phenotypic characters not present in the parental species used. For example, the following lines were identified and stabilized: appressed plant types with an acute leaf angle giving a cauliflower type of appearance to the plant, lines with high leaf area index, early-maturing tall and dwarf types, late-maturing tall and dwarf types, and lines with dense and sparse arrangement of leaves, small to very large leaves, small to large heads, short and long internodes, pigmented stems, and pale green to dark green leaves with variations in lamina structure. The stable pre-breeding materials developed using diploid annuals are serving as a source of maintainers or restorers for different cytoplasms and a source of resistance to rust and downy mildew, and represent material of interest for utilization in the sunflower breeding programme (Sujatha and Prabakaran 2004; Sujatha 2006). Pre-breeding material is generally selected and incorporated into breeding programmes based on morphological variation. While the value of morphological traits for evaluation varies according to the intended use of the material, knowledge of the level of genetic diversity in the pre-breeding material is important for selection of parental materials that can maximize the gain from selection.

In sunflower, several marker systems have been employed for assessment of genetic diversity in cultivated germplasm and wild sunflowers. Allozyme and RAPD polymorphisms were insufficient for distinguishing between closely or distantly related germplasm accessions in sunflower (Rieseberg and Seiler 1990; Rieseberg et al. 1993; Arias and Rieseberg 1995). Genetic diversity in wild and cultivated sunflower has been assessed using molecular markers such as RFLPs (Berry et al. 1994; Gentzbittel et al. 1994), AFLPs (Peerbolte and Peleman 1996; Gedil et al. 2001), and microsatellites (Dehmer and Friedt 1998; Yu et al. 2002; Burke et al. 2002; Tang et al. 2002, 2003; Tang and Knapp 2003). Berry et al. (1994), Gentzbittel et al. (1994), Rieseberg et al. (1993), and Arias and Rieseberg (1995) assessed allelic diversity in elite inbred lines using RFLPs and RAPDs, and DNA marker polymorphism rates ranged from 10% to 36% per cross. Sunflower-specific simple sequence repeats (SSRs) are informative, highly reproducible, and demonstrate a high degree of allelic variation (Yu et al. 2002, Burke et al. 2002; Tang et al. 2002, 2003; Tang and Knapp 2003). The abundance, codominant nature, high level of polymorphism, ready transferability, and ease of genotyping of SSRs make them an excellent molecular system for genetic diversity analysis. A total of 2968 SSR markers, including 1707 mapped markers, are available for molecular breeding and genomics research in sunflower.

The aim of the present study was to characterize advanced-generation lines derived from interspecific hybrids for cytomorphological relationships at diakinesis and to assess the genetic diversity to identify divergent lines for use in sunflower breeding programmes.

Materials and methods

Plant material

The plant material for this study was derived from interspecific hybrids between cultivated and wild diploid annual sunflowers, viz., *H. annuus* (wild), *H. argophyllus*, *H. petiolaris*, and *H. debilis.* Tri-species hybrids involving *H. argophyllus* and *H. annuus* with a sunflower cultivar (*H. annuus* 'Morden') were aimed at combining desirable traits such as rust and downy mildew resistance from the two diploid wild species with improved oil quality, yield, and other traits of agronomic value from cultivated sunflower. Branching and purple disc are undesirable traits in commercial sunflower and hence selection was done against these traits in each generation after a limited number of backcrosses with the cultivar as the recurrent pollen parent. A large number of lines with distinct phenotypes and other desirable traits have been selected, and the stabilized lines in the advanced generation are maintained through sib-mating.

Phenotypic evaluation

Forty backcross-derived inbred lines were selected from 250 lines based on initial diversity analysis of quantitative characters evaluated for 2 years. The 250 lines were initially subjected to cluster analysis based on Euclidian distance and the 40 most divergent lines were selected, representing 15% diversity from each cluster. Euclidian distance is the shortest geometric distance between two points in multidimensional space and is computed as

$$\text{distance}(x, y) = [\Sigma_i (x_i - y_i)^2]^{1/2}$$

The pedigree and morphological characteristics of the inbred lines are presented in Table 1. The 40 advanced lines and the 2 controls (Morden and CO-4) were grown in a 6 × 7 rectangular lattice with 3 replications. Morden is an early-flowering dwarf variety, while CO-4 is a medium-duration tall variety. A plot size of 4 rows of 4.2 m was adopted, with 0.60 m between rows and 0.30 m between plants within a row. Observations of 9 quantitative traits (days to flowering, days to 100% flowering, leaf chlorophyll (measured using a SPAD meter), plant height (cm), head diameter (cm), seed yield (g/plant), 100 seed mass (g), stem mass (g), and oil content (%)) and 14 qualitative traits (head angle at maturity; head size and shape; stem pigmentation and thickness; leaf size, shape, margin, base, and colour; petiole type and leaf habit of petiole; ray floret colour and shape) were recorded following IPGRI (1985) descriptors and Ranganatha et al. (2007).

Table 1. Morphological characteristics and pedigree of the prebred sunflower lines used in molecular characterization with microsatellite markers.

PS No.	Pedigree	Morphological characters
1014	BC_4F_8 of *H. argophyllus* × Morden	Early flowering, medium height, high leaf area index (LAI)
1018	BC_4F_8 of *H. argophyllus* × Morden	Medium height, thick stem, long internodes
1028	BC_4F_8 of *H. argophyllus* × Morden	Deeply serrated leaf, early, small leaves
1041	BC_4F_8 of *H. argophyllus* × Morden	Late flowering, small leaves, deeply serrated and many fewer leaves
1053	BC_4F_8 of *H. argophyllus* × Morden	Short
1068B	BC_4F_8 of *H. argophyllus* × Morden	Medium, better plant type, sparse arrangement of leaves, large heads, less susceptible to *Alternaria*
1082	BC_4F_8 of *H. argophyllus* × Morden	Normal, small but many leaves, deeply serrated leaves, thin stem
1084	BC_4F_8 of *H. argophyllus* × Morden	Thick stem, medium tall
1088	BC_4F_8 of *H. argophyllus* × Morden	Medium tall, deeply serrated leaves
1091	BC_4F_8 of *H. argophyllus* × Morden	Medium, large round leaves, long internodes, thick stem
44	BC_4F_8 of *H. petiolaris* × Morden	Short, high LAI
73	BC_4F_8 of *H. petiolaris* × Morden	Tall, long internodes
2001	BC_4F_8 of *H. petiolaris* × Morden	Short and fewer leaves, dwarf, early
2005	BC_4F_8 of *H. petiolaris* × Morden	Thick stem, tall, high LAI with large leaves, linear ray florets
2011	BC_4F_8 of *H. petiolaris* × Morden	Tolerant to rust
2013	BC_4F_8 of *H. petiolaris* × Morden	Zigzag and wavy stem, ovate leaves, lodging susceptible, early
2014	BC_4F_8 of *H. petiolaris* × Morden	Thick stem, tall, large leaves
2020	BC_4F_8 of *H. petiolaris* × Morden	Prominent venation of the leaf, tall, long petiole, sparsely arranged leaves
2033	BC_4F_8 of *H. petiolaris* × Morden	Round cup-shaped leaves, long internodes
2038	BC_4F_8 of *H. petiolaris* × Morden	Medium tall, stem-bending type
2046	BC_4F_8 of *H. petiolaris* × Morden	Tall, thick stem, large leaves
2047a	BC_4F_8 of *H. petiolaris* × Morden	Large thick leaves, high LAI and clustering, dense arrangement of leaves
2048	BC_4F_8 of *H. petiolaris* × Morden	Early flowering
2058	BC_4F_8 of *H. petiolaris* × Morden	Tall, large leaves, medium thick stem, peculiar leaf arrangement
2062	BC_4F_8 of *H. petiolaris* × Morden	Dark green crinkled leaf, normal height, thick stem
3004	BC_3F_8 of *H. annuus* (wild) × Morden	Dwarf, cauliflower type
3007	BC_3F_8 of *H. annuus* (wild) × Morden	Cauliflower type of leaf arrangement, late-maturing type
3009	BC_3F_8 of *H. annuus* (wild) × Morden	Cauliflower type of leaf arrangement
3036	BC_3F_8 of *H. annuus* (wild) × Morden	Tall, regularly serrated cordate leaf with pubescence and thick petiole
4015	BC_3F_8 of (*H. argophyllus* × *H. annuus* (wild)) × Morden	Early, fewer leaves, angle of flower head bending >135°, short
4020	BC_3F_8 of (*H. argophyllus* × *H. annuus* (wild)) × Morden	Normal, fewer leaves, pigmented petiole, not very productive, thin stem, small heads
4022	BC_3F_8 of (*H. argophyllus* × *H. annuus* (wild)) × Morden	Medium height, thick leaf, slightly turned
4061	BC_3F_8 of (*H. argophyllus* × *H. annuus* (wild)) × Morden	Betel vine leaf type, pale green, soft ovate leaves, capitula covered with upper leaves, short
4066	BC_3F_8 of (*H. argophyllus* × *H. annuus* (wild)) × Morden	Medium height, large leaves, optimum-size heads
4083	BC_3F_8 of (*H. argophyllus* × *H. annuus* (wild)) × Morden	Light green leaves, tall, late maturing, large leaves, long petiole, tree-type appearance
4093	BC_3F_8 of (*H. argophyllus* × *H. annuus* (wild)) × Morden	Tall, high LAI, long thick petioles, thick stem
4096	BC_3F_8 of (*H. argophyllus* × *H. annuus* (wild)) × Morden	Round ray florets, soft leaf, less serrated, spiral phyllotaxy, small heads, less susceptible to *Alternaria*
4111	BC_3F_8 of (*H. argophyllus* × *H. annuus* (wild)) × Morden	Short, fewer leaves, late maturity
4114	BC_3F_8 of (*H. argophyllus* × *H. annuus* (wild)) × Morden	Medium, large cup-shaped leaves
5016	BC_4F_8 of CMS 234A × *H. debilis*	Restorer for *H. argophyllus* cytoplasm. Highly uniform, small leaves, typical head position, wavy leaf, late maturity
Morden	Open-pollinated variety	Dwarf, early-maturing variety
CO-4	Open-pollinated variety	Medium duration, medium-tall variety

Table 2. Meiotic chromosome associations at diakinesis of backcross-derived inbred lines of sunflower.

Cross combination	Inbred line	Chromosome association at diakinesis				Mean chromosome association	Pollen fertility (%)
		I	II	III	IV		
H. argophyllus × *H. annuus*	PS 1035	0–2	14–17	—	0–1	0.35 I + 16.13 II + 0.35 IV	91.7
	PS 1043	0–6	13–17	—	0–2	0.35 I + 15.6 II + 0.61 IV	90.4
	PS 1056	—	13–17	—	0–2	15.7 II + 0.65 IV	95.4
	PS 1061	—	15–17	—	0–1	16.4 II + 0.3 IV	93.6
	PS 1069	—	13–17	—	0–2	16.2 II + 0.4 IV	80.2
H. petiolaris × *H. annuus*	PS 2002	0–2	12–13	0–1	0–2	0.15 I + 15.35 II + 0.05 III + 0.75 IV	89.6
	PS 2010	0–6	10–17	0–2	0–2	1.35 I + 14.75 II + 0.25 III + 0.55 IV	87.5
	PS 2023	0–2	11–17	0–2	0–3	0.1 I + 15.5 II + 0.1 III + 0.65 IV	89.4
	PS 2036	0–4	13–17	—	0–2	0.7 I + 15.65 II + 0.5 IV	90.1
	PS 2040	—	13–17	—	0–2	16.0 II + 0.5 IV	94.5
H. annuus (wild) × *H. annuus*	PS 3003	0–4	13–17	—	0–1	0.2 I + 16.2 II + 0.35 IV	93.6
	PS 3009	—	15–17	—	0–1	16.2 II + 0.4 IV	95.4
	PS 3011	—	13–17	—	0–1	16.3 II + 0.3 IV	96.2
	PS 3015	—	15–17	—	0–1	16.4 II + 0.3 IV	92.1
	PS 3017	—	15–17	—	0–1	16.5 II + 0.25 IV	89.4
H. argophyllus × *H. annuus* (wild) × *H. annuus*	PS 4002	0–2	14–17	—	0–1	0.2 I + 16.0 II + 0.45 IV	93.4
	PS 4005	—	13–17	—	0–2	15.8 II + 0.6 IV	92.8
	PS 4012	—	13–17	—	0–2	16.1 II + 0.45 IV	91.5
	PS 4042	—	13–17	—	0–2	15.8 II + 0.6 IV	90.4
H. annuus × *H. debilis*	PS 5014	0–2	11–17	—	0–3	0.4 I + 14.8 II + 1.0 IV	93.2
	PS 5017	0–2	13–17	—	0–2	0.2 I + 15.7 II + 0.6 IV	86.4
	PS 5021	0–2	13–17	0–1	0–2	0.35 I + 16.05 II + 0.05 III + 0.35 IV	83.2
	PS 5023	—	13–17	—	0–2	16.0 II + 0.5 IV	91.7
	PS 5025	0–4	14–17	—	0–1	0.3 I + 15.85 II + 0.5 IV	88.4

Cytology

For cytological analysis, 24 prebred lines comprising 4 or 5 lines from each of the cross combinations were used. Young flower buds of appropriate size were fixed in Carnoy's II solution (6:3:1 absolute ethanol : chloroform : glacial acetic acid) for 24 h and stored in 70% alcohol at 4 °C. Pollen mother cell (PMC) smears were prepared using 1% acetocarmine, and chromosomal associations of at least 100 PMCs were recorded after destaining the slides with 45% acetic acid. For study of pollen fertility, pollen was stained in a 1:1 mixture of 1% acetocarmine and glycerol. The fully stained and round pollen grains were scored as fertile, while shriveled and unstained grains were recorded as sterile.

DNA extraction and SSR analysis

For each line, leaves were harvested from 20 field-grown 6-week-old plants and bulked for DNA isolation. DNA was extracted following the CTAB method (Webb and Knapp 1990) and quantified on 0.8% agarose using a known concentration of λ DNA as standard.

Using information on marker distribution on a genetic linkage map, 98 SSR primers of the ORS series (Knapp 2004) and 20 SSRs from Paniego et al. (2002) were selected. Polymerase chain reaction (PCR) was performed according to Yu et al. (2002). The codes for the ORS series were the original codes, while the SSRs of Paniego et al. (2002) were designated SF1 to SF20. The reaction mix (20 µL) contained 20 ng of DNA template, 5.5 pmol of each primer, 2.5 mmol/L $MgCl_2$, 125 µmol/L each dNTP, 1× PCR buffer, and 0.8 U of *Taq* DNA polymerase (Genei, Bangalore, India). The PCR amplification profile included an initial denaturation step at 94 °C for 2 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min in a GeneAmp PCR System 9700 thermocycler (Perkin Elmer – Applied Biosystems). Annealing temperature varied between 51 and 60 °C for the primers used. The PCR-amplified products were run on 3.5% agarose (Genei, Bangalore, India) in 1× Tris-acetate EDTA buffer with ethidium bromide (50 ng/mL) for 2 h at 70 V for detection of allelic variations. A 50/100 bp ladder was used for measuring the allele size and an accurate size was determined using Quantity One software (Bio-Rad).

Data analysis

Presence or absence of a fragment was coded in a binary data matrix as 1 or 0, respectively. In the case of primers revealing more than one allele, PCR amplifications were repeated. A band was considered unique if it was present in one line but absent in the remaining genotypes. Pair-wise genetic similarities (S_{ij}) between accessions i and j were estimated using the similarity coefficient of Nei and Li (1979), $S_{ij} = 2N_{ij}/(N_i + N_j)$, where N_{ij} is the number of bands in common between any two accessions and S_{ij} may range from 0 (no common bands) to 1 (identical band profiles for the two accessions). S_{ij} values were used to estimate genetic dissimilarity ($D_{ij} = 1 - S_{ij}$), and the D_{ij} values were used to determine the relationships among accessions using principal

coordinate analysis (PCoA) (Sneath and Sokal 1973). The polymorphism information content (PIC) was calculated according to Botstein et al. (1980):

$$\mathrm{PIC} = 1 - \sum_{i=1}^{k} p_i^2 - \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} 2p_i^2 p_j^2$$

where k is the number of alleles for a given marker and p_i and p_j are the allelic frequencies. The Mantel test of significance was used to determine the goodness of fit for the correlation between phenotypic traits and molecular data. All computations were performed using the statistical computing package GenStat release 6.1 (Payne 2002) and NTSYS pc version 2.20 (Applied Biostatistics Inc., Setauket, New York). For association mapping, multidimensional scaling was carried out in NTSYS pc for the 42 inbred lines based on morphological data, and stepwise regression analysis was carried out in GenStat using the phenotypic data.

Results

Cytomorphological diversity

We were interested to know the influence of leftover wild genome in the stabilized lines on meiotic chromosome pairing and pollen fertility. Diakinesis is the ideal stage for the study of chromosome associations. Irrespective of the cross combination, the backcross-derived inbred lines showed normal meiosis in 50%–70% of the PMCs and formation of 17 bivalents (Table 2). Among the several bivalent shapes, ring, rod, and open-ring bivalents occurred more frequently than other configurations such as '8', 'V', loose chain, or bracket (Fig. 1). The inbred lines frequently showed the presence of quadrivalents in addition to normal bivalents. Quadrivalents were observed in all the lines at frequencies ranging from 1 to 3 (Table 2; Fig. 1). Among the 24 prebred lines, several showed two quadrivalents during diakinesis. In contrast, a third quadrivalent was reported in PS 2023 and PS 5014. The lines PS 2010, PS 2023, PS 2002, and PS 5021 occasionally showed trivalents during meiosis. In this study, the frequency of univalents ranged from 0 to 6, with lines PS 1043 and PS 2010 showing up to 6 univalents during diakinesis. Many lines showed pollen fertility comparable with that of cultivated sunflower, while a few lines, such as PS 1069, showed low pollen fertility (80.2%).

Molecular characterization

Of the 118 SSR primers tested, 100 produced 204 amplicons of the expected size, of which 199 (97.5%) were polymorphic. The primers ORS333, ORS334, ORS378, and ORS396 failed to produce amplification products; ORS163, ORS237, ORS244, ORS328, ORS447, and SF15 produced multiple bands that were difficult to interpret; ORS200, ORS240, ORS257, and ORS426 failed to produce amplicons of reference allele size; and ORS235, ORS258, ORS295, and ORS384 produced monomorphic bands. The number of alleles per locus ranged from 2 to 5 (Fig. 2). The PIC for polymorphic SSRs ranged from 0.05 to 0.575, with an average of 0.31 (Table 3). ORS169, SF11, and SF14 generated a maximum of 5 alleles. The polymorphic microsatellites produced an average of 2.04 alleles/locus.

Fig. 1. Chromosome associations observed at diakinesis in backcross-derived inbred lines of sunflower: (*a*) 17 II in PS 3017, (*b*) 16 II + 2 I in PS 1043, (*c*) 3 IV + 11 II in PS 5014, (*d*) 1 IV + 14 II + 2 I in PS 3003, (*e*) 3 IV + 1 III + 9 II + 1 I in PS 2023, (*f*) 3 IV + 10 II + 2 I in PS 5014, (*g*) 2 IV + 13 II in PS 1056, and (*h*) 3 IV + 9 II + 4 I in PS 2023.

Unique alleles

Of the 118 tested pairs of primers, 27 produced 46 unique alleles in 18 inbred lines (Table 4). Each of these 27 markers could distinguish 1 to 3 alleles. The marker ORS256 could distinguish 4 genotypes, detecting unique alleles in 2 inbred lines derived from the cross involving *H. petiolaris* (PS 2047a and PS 2048) and 2 lines derived from the trispecific cross (PS 4022 and PS 4066).

Preliminary data on the utility of SSRs for trait association analysis (Table 5) revealed positive and significant association of microsatellites with traits such as days to flowering and head diameter. The microsatellite marker $SF2_{153}$ was found to be significantly and positively correlated with days to flowering.

The genetic dissimilarity (D_{ij}) among the 42 lines ranged from 0.143 to 0.486, with an average of 0.311 (Table 6). The maximum genetic distance based on molecular characterization was found between PS 2020 and PS 1053 and between PS 4066 and PS 2048, while the genetic distance between the two commercial genotypes (CO-4 and Morden)

Fig. 2. Allelic variation in backcross-derived inbred lines of sunflower with SSR primer ORS202 (M, 100 bp DNA ladder; 1–42, samples).

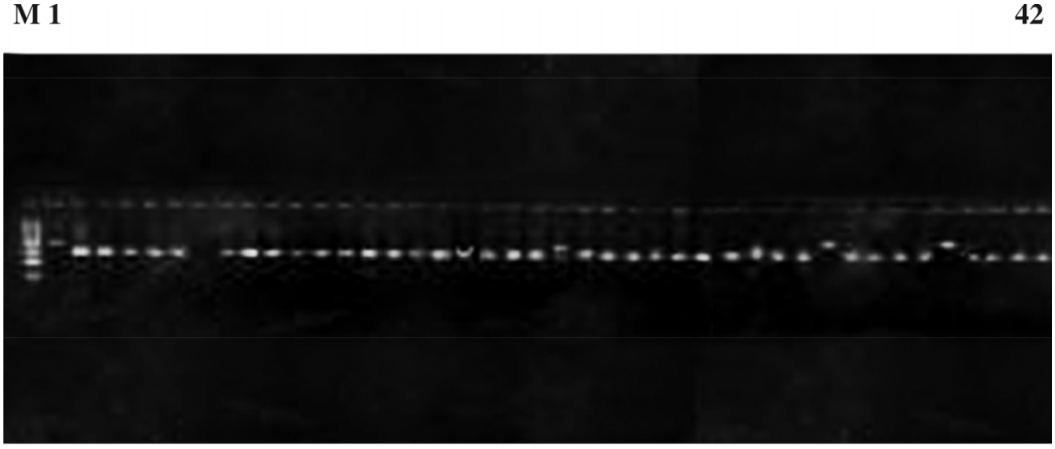


Table 3. PIC values for selected SSR primers.

Primer	PIC	Primer	PIC
ORS12	0.5006	ORS358	0.1886
ORS31	0.5013	ORS364	0.1737
ORS70	0.1623	ORS366	0.1351
ORS90	0.5068	ORS351	0.0526
ORS123	0.5011	ORS372	0.3556
ORS124	0.5747	ORS391	0.0908
ORS134	0.3926	ORS418	0.5392
ORS142	0.4860	ORS426	0.4791
ORS151	0.4702	ORS462	0.4441
ORS154	0.5095	ORS468	0.1141
ORS166	0.132	ORS481	0.5035
0RS169	0.5649	ORS485	0.0714
ORS170	0.4277	SF2	0.1505
ORS176	0.3704	SF3	0.1726
ORS185	0.0597	SF4	0.1028
ORS187	0.1024	SF5	0.3869
ORS188	0.4805	SF6	0.4261
ORS200	0.0635	SF7	0.1999
ORS202	0.2369	SF10	0.4442
OR6216	0.0667	SF11	0.2149
ORS243	0.3669	SF13	0.5098
ORS256	0.1786	SF14	0.3426
ORS307	0.5057	SF16	0.2571
ORS317	0.3075	SF19	0.1382
ORS344	0.2182	SF20	0.0455

was low. The mean genetic distance based on morphological characteristics (pooled for the dry and rainy seasons) between a specific line and the other 41 lines ranged from 0.006 to 0.395. The genetic distance based on phenotypic evaluation was low for PS 2062 × PS 2005 and maximum for PS 4083 × PS 4015. The most diverse inbred lines were PS 2048 ($\breve{D}$ = 0.406) and PS 1053 ($\breve{D}$ = 0.403), while PS 1091 ($\breve{D}$ = 0.258) showed the least diversity. Morphologically the lines diverged at 0.84 similarity, while at the molecular level they separated at a distance of 0.59.

Cluster analysis and principal coordinate analysis did not reveal distinct groupings. The clustering pattern based on molecular marker–based genetic distances was not significantly correlated with that based on morphological characters. A dendrogram based on the data pooled from the phenotypic and molecular analyses separated the lines into 4 clusters, and lines PS 4083 and PS 4093 (high LAI and tall, tree-like appearance) formed a distinct cluster (Fig. 3). Line PS 3009 has a cauliflower type of appearance, PS 4061 has a pale green betel vine type of leaves, and PS 4083 and PS 4093 have a small tree-like appearance with thick stems and densely arranged large dark green leaves. The correlation between the phenotypic traits and molecular data based on the Mantel statistic was not significant (r = 0.08860).

Discussion

The genomes of the diploid annual wild sunflowers constituting the primary gene pool are considered not similar but closely related to that of cultivated sunflower (Chandler et al. 1986). Higher chromosome associations such as trivalents and quadrivalents during meiosis have been reported in F_1 interspecific hybrids involving cultivated and wild diploid species of *Helianthus* (Whelan 1979; Whelan and Dorrell 1980; Chandler et al. 1986; Narkhede et al. 1986). This led to the conclusion that the genomes of these species in the primary gene pool differ from each other by a limited number of reciprocal translocations.

Georgieva-Todorova (1984) considered autosyndesis of chromosomes to be the cause of ring bivalents and heteromorphic nature (formed by conjugation between homoeologous chromosomes) to be the cause of more frequent rod bivalents. Exchange of unequal chromatin segments between nonhomologous chromosomes (translocation of chromosomes) may be a reason for the formation of quadrivalents (Chandler et al. 1986; Narkhede et al. 1986). A possible explanation for the differences between the present study and previous reports on interspecific hybrids of wild diploid species may be intraspecifc structural polymorphism. It is of interest that hybrids for which discrepancies exist involved either *H. petiolaris* or *H. debilis* as parent. Occasionally, secondary or nonhomologous association of chromosomes can give spurious results in the study of translocation heterozygosity in interspecific hybrids (Chandler et al. 1986). The presence of quadrivalents at the diakinesis stage in interspecific hybrids is well documented (Chandler et al. 1986). Narkhede et al. (1986) reported a single quadrivalent in the interspecific hybrid between sunflower and *H. argo-*

Table 4. Unique alleles in backcross-derived inbred lines of sunflower.

Cross combination	Primers giving unique alleles for the cross combination	Frequency of unique alleles	PS No.	Unique alleles
H. argophyllus × *H. annuus*	2	0.4	1018	$ORS358_{400}$, $SF4_{250}$, $SF16_{210}$, $SF19_{190}$
			1053	$ORS344_{210}$
			1082	$ORS176_{445}$, $ORS485_{350}$, $SF11_{450}$
H. petiolaris × *H. annuus*	10	1.4	44	$SF11_{410}$
			2001	$ORS186_{103}$, $ORS364_{190}$
			2005	$ORS166_{400}$, $ORS166_{200}$, $ORS364_{190}$, $ORS391_{400}$
			2014	$SF14_{140}$, $SF14_{200}$, $SF20_{420}$
			2038	$ORS342_{250}$, $ORS342_{200}$, $SF16_{480}$, $SF19_{150}$
			2046	$ORS371_{190}$
			2047a	$ORS256_{200}$, $SF7_{300}$
			2048	$ORS256_{190}$, $SF4_{360}$, $SF4_{450}$
			2058	$ORS200_{380}$, $ORS364_{170}$
			2062	$ORS216_{280}$, $ORS364_{170}$, $ORS372_{180}$, $SF11_{500}$, $SF14_{140}$, $SF14_{500}$, $SF16_{220}$
H. annuus (wild) × *H. annuus*	1	0.5	3007	$SF14_{150}$
			3009	$ORS188_{370}$
H. argophyllus × *H. annuus* (wild) × *H. annuus*	5	0.5	4061	$ORS185_{316}$, $ORS187_{277}$, $ORS344_{180}$, $ORS358_{260}$, $SF7_{260}$
			4066	$ORS187_{277}$, $ORS256_{250}$, $ORS366_{220}$, $SF2_{400}$, $SF6_{450}$
			4083	$SF2_{153}$

phyllus and 2 quadrivalents in the interspecific hybrid between *H. annuus* and *H. debilis* and suggested that the genomes differed by 1 and 2 reciprocal translocations, respectively. It is expected that interspecific hybrids will possess a genome contributed equally by the 2 parents in order to show multivalent configurations. But in recombinant inbred lines, after 2–3 backcrosses a significant portion of the wild genome is replaced by the cultivar genome; therefore, the chance of multivalent formation is reduced. However, the prebred lines, which are stabilized interspecific derivatives, also showed quadrivalents. Hence, it may be concluded that recombinant inbred lines developed through interspecific hybridization possess a very large spectrum of variability due to a high degree of recombination between nonhomologous chromosomes of the parental genomes, as revealed by the presence of 1–3 translocations.

The formation of trivalents may be attributed to loose or improper pairing of quadrivalents, which could lead to the dissociation of quadrivalents into one trivalent and a univalent (Narkhede et al. 1986). Chandler et al. (1986) considered secondary associations to be the cause of formation of univalents. Secondary associations occur when homologous pairing is incomplete in individuals such as haploids and interspecific hybrids. Since *Helianthus* is considered to have an ancient tetraploid origin, secondary associations may also be the source of univalent formation.

Reduced pollen fertility (80.2%) in PS 1069 can be correlated with the high frequency of chromosome bridges and laggards that was observed in anaphase I. Reduced pollen fertility in interspecific hybrids has been reported (Georgieva-Todorova 1984; Narkhede et al. 1986; Espinasse et al. 1995). Since an interspecific hybrid has genomes from both parents, the presence of a wild genome in the hybrid is enough to cause meiotic irregularities that lead to reduced pollen fertility. However, continued backcrosses with cultivated sunflower eventually eliminate wild characters, and stabilization of meiosis should have occurred more rapidly, as reported in *H. maximiliani* × *H. annuus* interspecific hybrids by Whelan and Dorrell (1980).

The number of alleles detected will depend on the diversity of the germplasm sampled and also on the informativeness of the marker or primer set. The PIC scores in this study were not significantly different from those reported earlier (0.17–0.49, Gedil 1999; 0.31–0.55, Yu et al. 2002). In the study of Paniego et al. (2002), the polymorphic microsatellites produced an average of 3.5 alleles/locus and an average PIC of 0.55. This variation in PIC could be due to the limited number of microsatellites (100) used in our study, in contrast to the study of Paniego et al. (2002), in which 271 microsatellites were used. Furthermore, we checked the allelic variation on agarose gels with ethidium bromide staining, unlike the study of Paniego et al. (2002), where denaturing polyacrylamide gels with silver staining were used. Most studies of SSRs in sunflower have employed automated multiplex PCR using a multicolor assay based on fluorophores that reveal polymorphism more discretely (Burke et al. 2002; Tang et al. 2002; Yu et al. 2002).

Six primers produced multiple bands. Paniego et al. (2002) made a similar observation and attributed such complex patterns to the heterogeneity or heterozygosity of the inbred lines or the presence of 2 (or more) duplicated microsatellite loci. However, only 6 of the 118 microsatellites (5.1%) tested in this study gave evidence of heterozygosity and (or) heterogeneity, suggesting a sufficient degree of inbreeding in the inbred lines analyzed in this study.

The present study reveals that microsatellites are useful not only in discriminating lines derived from the same pedigree but also in identification of unique alleles in backcross-

Table 5. Association analysis using molecular marker information.

	Rainy season				Post rainy season				Pooled			
Trait	Marker	β ± SE	*t* prob	R^2	Marker	β ± SE	*t* prob	R^2	Marker	β ± SE	*t* prob	R^2
Days to first flower	$SF2_{400}$	18.97±4.881	0.0004	25.59								
Days to 50% flowering	$SF2_{400}$	21.86±4.766	0.0	32.83					$SF2_{400}$	17.64±4.644	0.0005	24.67
Oil content	$ORS166_{200}$	–9.53±2.327	0.0002	27.76	SF10	4.31±1.066	0.0002	27.18	$ORS166_{200}$	–7.82±1.763	0.0001	31.28
Head angle	SF16	–1.63±0.394	0.0002	28.32								
Head diameter					$SF11_{600}$	8.58±1.459	0.0	45.02	SF10	1.81±0.482	0.0005	24.24
Seed mass									SF10	8.66±2.131	0.0002	27.44

derived inbred lines. Paniego et al. (2002) demonstrated the potential of SSR markers in identification of inbred lines of sunflower and assessment of their distinctness and genetic diversity. The presence of unique alleles is an indication of a high rate of genetic rearrangement. Unique alleles are significant because they can be diagnostic of a particular inbred line or regions of the genome specific to a particular type of genotype (Senior et al. 1998). The primer ORS256 could distinguish 4 genotypes in this study. This marker was highly polymorphic and had 15 alleles with a PIC score of 0.93 (Yu et al. 2002). The occurrence of the maximum number of unique alleles in the cross combination involving *H. petiolaris* indicates the potential of this species to serve as a reservoir of novel alleles for sunflower improvement. Cytological investigations carried out in the present study revealed a high degree of structural polymorphism, with a higher number of multivalents in interspecific hybrids involving *H. petiolaris* or *H. debilis* as parent. *Helianthus petiolaris* has been widely exploited as a source of sterile cytoplasm on which entire sunflower hybrid breeding programmes are based, and this study reveals the usefulness of this species in widening the genetic base of cultivated sunflower. The allelic diversity in the lines derived from the trispecific cross was low compared with that in lines derived from *H. petiolaris.* Although lines derived from crosses involving *H. debilis* showed a high degree of chromosomal rearrangements, not much information could be drawn from molecular characterization, as it involved only one line. Allelic diversity in Native American land races and wild populations of cultivated sunflower showed that wild populations are a wellspring of genetic diversity (Tang and Knapp 2003). Allelic diversity based on allozyme analysis revealed a higher number of unique alleles in wild populations than in domesticated sunflower (Rieseberg and Seiler 1990). Tang and Knapp (2003) found a gradual but dramatic (33-fold) narrowing of allelic diversity from taxon-specific alleles of the wild population to the elite inbred lines.

In this study, null alleles were detected. RFLP and SSR markers are rarely dominant in crop plants (Akkaya et al. 1992; Liu et al. 1996). However, several studies have proved that sunflower is an exception to this observation; these studies reported that 9% to 30% of the SSRs and RFLPs produced null alleles among elite inbred lines (Yu et al. 2002; Berry et al. 1994; Gentzbittel et al. 1994; Jan et al. 1998).

Cluster analysis and principal coordinate analysis were used for visualization of estimates of genetic distance. Cluster analysis of these lines revealed a lack of structure, and there were small clusters with no clear grouping. Presumably this could be due to differences in the pedigree of the lines and the selection of parents with contrasting and common traits, such as dwarf with early and late maturity, tall with both maturity groups, and so on. Most other genetic analyses have been based on market classes, fertility/restorer lines, and geographical entities (Yu et al. 2002).

The presence of a high degree of chromosomal rearrangements, the high frequency of unique alleles, and the maximum genetic distance revealed by these lines are indicative of the usefulness of *H. petiolaris* in introgressive breeding. The diverse genotypes of Argentine origin are also a result

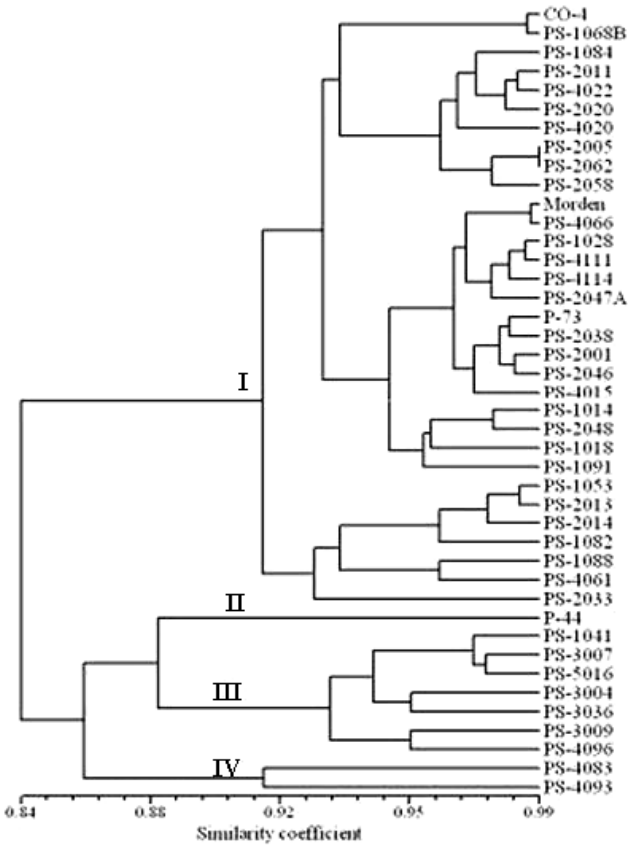
Table 6. Molecular and phenotypic diversity and trait differences among selected lines derived from interspecific hybrids of sunflower.

	Genetic diversity		
Genotype combination	Molecular	Phenotypic	Major trait differences
PS 1053 / PS 4083	0.442	0.257	Dwarf / Tall, high LAI
PS 1068B / PS 1041	0.232	0.243	Large heads, high LAI / Small heads, low LAI
PS 1068B / PS 5016	0.244	0.237	Large heads / Small heads
PS 1091 / PS 1041	0.254	0.321	Large leaves / Small leaves
PS 1091 / PS 1082	0.217	0.218	Large leaves, thick stem / Small leaves, thin stem
PS 1091 / PS 3004	0.298	0.243	Long internodes / Appressed type
PS 1091 / PS 3007	0.286	0.301	Long internodes / Appressed type
PS 1091 / PS 4096	0.253	0.239	—
PS 2001 / PS 1041	0.275	0.259	Early flowering / Late flowering
PS 2001 / PS 3004	0.318	0.214	Early flowering / Late flowering
PS 2001 / PS 3007	0.264	0.252	Early flowering / Late flowering
PS 2001 / PS 3036	0.249	0.239	Dwarf / Tall
PS 2001 / PS 5016	0.267	0.226	Early flowering / Late flowering
PS 2048 / PS 1041	0.402	0.207	Early, high LAI / Late, low LAI
PS 2048 / PS 4083	0.324	0.255	Early flowering / Late flowering
PS 3004 / PS 73	0.326	0.238	Low test mass / High test mass
PS 4061 / CO-4	0.337	0.253	Late, betel vine type leaves / Normal, green
PS 3009 / CO-4	0.353	0.275	Appressed plant type / Normal
PS 3009 / Morden	0.371	0.223	Late flowering / Early flowering
PS 3009 / PS 73	0.361	0.251	Short internodes, low test mass / Long internodes, high test mass
PS 3009 / PS 1014	0.410	0.229	Late flowering / Early flowering
PS 3009 / PS 1018	0.342	0.305	Short internodes / Long internodes
PS 3009 / PS 1028	0.373	0.222	Late flowering / Early flowering
PS 3009 / PS 1068B	0.372	0.249	More leaves / Sparse arrangement of few leaves
PS 3009 / PS 1091	0.372	0.366	Short internodes / Long internodes
PS 3009 / PS 2001	0.341	0.280	Late, more leaves / Early, fewer leaves
PS 3009 / PS 2038	0.367	0.215	Straight stems / Bending of stems
PS 3009 / PS 2046	0.338	0.208	Small heads / Large heads
PS 3009 / PS 2048	0.476	0.256	Late flowering / Early flowering
PS 3009 / PS 2059	0.399	0.210	Dwarf / Tall
PS 3009 / PS 2062	0.396	0.220	Normal pale leaves / Dark green crinkled leaves
PS 3009 / PS 4015	0.369	0.250	Late flowering / Early flowering
PS 3009 / PS 4093	0.418	0.203	Short, cauliflower type / Tall, tree type
PS 3009 / PS 4111	0.362	0.249	More leaves / Fewer leaves
PS 3009 / PS 4114	0.362	0.225	Low test mass, late, medium tall, small heads / High test mass, early, tall, large heads
PS 4066 / PS 73	0.349	0.304	—
PS 4066 / PS 2048	0.481	0.045	Late maturity / Early maturity
PS 4083 / CO-4	0.256	0.268	—
PS 4083 / Morden	0.263	0.317	Late, tall / Early, short
PS 4083 / PS 73	0.358	0.379	High LAI / Medium-size leaves
PS 4083 / PS 1014	0.386	0.218	Tall, late / Medium, early
PS 4083 / PS 1018	0.351	0.259	Tall, short internodes / Dwarf, long internodes
PS 4083 / PS 1028	0.407	0.340	High LAI, large leaves, late / Small leaves, early
PS 4083 / PS 1053	0.442	0.257	Tall / Dwarf
PS 4083 / PS 1068B	0.369	0.256	Large leaves, more leaves / Sparse arrangement of leaves
PS 4083 / PS 1084	0.395	0.234	Tall / Medium
PS 4083 / PS 1091	0.315	0.365	Short internodes / Long internodes
PS 4083 / PS 2001	0.338	0.347	Tall, high LAI, late / Dwarf, low LAI, fewer leaves, early
PS 4083 / PS 2013	0.409	0.294	Late flowering / Early flowering
PS 4083 / PS 2014	0.346	0.276	Late flowering / Early flowering
PS 4083 / PS 2046	0.305	0.283	—
PS 4083 / PS 2047a	0.324	0.238	—
PS 4083 / PS 2048	0.420	0.255	Late flowering / Early flowering
PS 4083 / PS 4015	0.352	0.395	Tall, late / Early, short
PS 4083 / PS 4022	0.377	0.214	Tall / Medium
PS 4083 / PS 4061	0.358	0.202	Tall, dark green leaves / Short, betel vine type pale green leaves

Table 6 (*concluded*).

Genotype combination	Genetic diversity		Major trait differences
	Molecular	Phenotypic	
PS 4083 / PS 4066	0.362	0.276	Tall / Medium
PS 4083 / PS 4111	0.328	0.352	Tall / Medium
PS 4083 / PS 4114	0.299	0.307	Tall, late / Medium, early
PS 4083 / PS 5016	0.285	0.216	Large leaves / Small leaves
PS 5016 / CO-4	0.250	0.254	Small leaves / Medium leaves
PS 5016 / PS 73	0.253	0.231	Low test mass, medium / High test mass, tall
PS 5016 / PS 1018	0.265	0.232	High chlorophyll content, thin stem / Low chlorophyll content, thick stem
PS 5016 / PS 1068B	0.244	0.237	Low oil content, small heads / High oil content, large heads
PS 5016 / PS 1091	0.232	0.275	Low oil content, high chlorophyll content, thin stem / High oil content, low chlorophyll content, thick stem

Fig. 3. Dendrogram of 40 backcross-derived inbred lines of sunflower along with 2 commercial cultivars (CO-4 and Morden) based on pooled genetic distance as measured by phenotypic variation in two seasons and allelic variation determined using SSR markers.



of intensive utilization of wide interspecific crossing with diploid annual sunflowers (Paniego et al. 2002).

The earlier genetic maps were constructed using F_2 progeny from a cross between oilseed sterility maintainer lines, RILs from crosses within proprietory oilseed fertility restorer lines, and RILs from crosses between public confectionery and oilseed fertility restorer lines (Gedil 1999; Yu et al. 2002; Tang et al. 2002). The SSR markers placed on these three elite × elite cross maps represent the subset with the greatest utility for molecular breeding in the highly selected and domesticated gene pools on which the commercial hybrid breeding programmes are based. The materials used in the above-mentioned studies represent only part of the overall variability. The studies of Tang and Knapp (2003) and Burke et al. (2002) emphasize the need for use of progeny from elite × wild crosses to increase the density of the molecular genetic linkage map of sunflower, because allelic variability declines 33-fold from wild to domesticated cultivars. In the present study we have identified several unique alleles in genotyping 42 lines with 118 SSR primers on agarose gels. There is a distinct possibility of identifying several unique alleles in the 250 lines developed if they are genotyped using a larger number of mapped SSR markers on semi-automated or automated systems to facilitate increasing the density of the genetic map.

For quantitative characters such as yield, heterotic response is expected to increase with parental genetic distance (Melchinger 1999). Use of backcross-derived lines facilitates the detection of linkages. Cytomorphological studies coupled with molecular characterization using mapped SSR markers can help to define the genetic architecture of the germplasm and to identify alleles associated with key phenotypic traits. This study shows that the molecular variation is high and the backcross-derived inbred lines with the same phenotype show high allelic diversity. The inbred lines in the present study were derived from interspecific crosses of diploid annual species. The lines were defined with chromosomal associations at diakinesis and microsatellite markers. This molecular diversity analysis will assist sunflower breeders in inbred line development and facilitate identification of well-defined heterotic groups. Further, all the stabilized inbred lines will be characterized using FISH techniques to demonstrate the extent of introgression in terms of the number and size of the introgressed fragments, according to Feng et al. (2005). The lines will also be subjected to molecular classification for identification of new sources of alleles for broadening the genetic base of sunflower.

Acknowledgements

The authors thank the Project Director, Directorate of Oilseeds Research, for providing the facilities for carrying out the research work and Ms. M. Tarakeswari for her technical help in SSR analysis.

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